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Solar photo-Fenton oxidation followed by adsorption on activated carbon for the minimisation of antibiotic resistance determinants and toxicity present in urban wastewater

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ABSTRACT

This work evaluated the removal of a mixture of antibiotics from urban wastewater, by a combined process consisting of solar photo-Fenton (SPF) followed by granular activated carbon (GAC). The effects of the SPF process were investigated at a toxicological, microbiological and genomic level, using species of plants and aquatic organisms, bacteria, antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs). The results demonstrated that SPF could completely degrade antibiotics present in two types of effluent deriving from a conventional activated sludge system (CAS) and a membrane bioreactor (MBR), operated at the optimum oxidant dose ($[H_2O_2]_{CAS}$ = 100 mg/L; $[H_2O_2]_{MBR}$ = 50 mg/L) and illumination time (t_{CAS} = 115 min; t_{MBR} = 111 min) at acidic pH (2.8–2.9). Moreover, total disinfection was achieved by the SPF process, as cultivable bacteria, including ARB, were inactivated after 60 min of treatment, disabling also the bacterial regrowth after 24 h of storage of the treated effluent. Furthermore, SPF was shown to be effective in degrading the cellular DNA of the effluent, which was reduced to the detection limit after 60 min of treatment in both effluents. The abundance of 16S rRNA was found to be preferentially decreased by the SPF in the MBR-treated effluent (8 folds) compared to the CAS-treated effluent (7.4 folds). The abundance of *bla*$_{OXA}$, *bla*$_{CTX-M}$, *qnrS*, *sul*1, and *tet*M genes was decreased to the limit of quantification after 60 min of SPF treatment in both effluents. However, the SPF treated flow resulted in increased toxicity, probably due to the oxidation of the dissolved effluent organic matter of the wastewater leading to the formation of toxic products. Therefore, SPF-treated samples collected at different time intervals (30, 60, 90, 120, and 180 min) were subjected to adsorption onto GAC (500 mg/L), and the removal of both the toxicity and the residual antibiotics remaining after SPF, was explored. The combined processes (30 min SPF; 15 min GAC) provided almost complete removal of toxicity and elimination of antibiotics, ensuring wastewater decontamination.

1. Introduction

The inclusion of some antibiotics in the revised Watch List drafted by the European Commission [1] and the appearance of new challenges in wastewater reuse applications regarding contaminants of emerging concern (CECs), including antibiotics, antibiotic-resistant bacteria (ARB), and resistance genes (ARGs), have demonstrated the need for the application of new and improved wastewater treatment technologies, able to effectively remove these microcontaminants from urban wastewater [2]. A potential alternative solution could be the utilization of advanced treatment technologies which can increase the removal of CECs and, consequently, enhance the quality of the effluents before their reuse or discharge into the environment.

Advanced chemical oxidation processes (AOPs) have attracted major scientific interest as a promising alternative for the degradation of antibiotics in wastewater [3]. They involve chemical reactions that generate highly reactive radical species, such as the hydroxyl radicals ($HO^{\cdot}$). Among the existing AOPs, Fenton process, which is based on the production of $HO^{\cdot}$ from the reaction between hydrogen peroxide and ferrous ions in acidic medium, can be accelerated when the solution is irradiated with light (photo-Fenton) and promotes the oxidation and mineralisation of organic contaminants. Some of the advantages of

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photo-Fenton are its high efficiency in degrading different types of organic substrates and the use of solar irradiation to stimulate the radicals' formation, resulting thus, in a low-cost application [4]. Solar photo-Fenton (SPF) has been shown to be capable of completely oxidizing various organic microcontaminants, while also providing sufficient disinfection of wastewater. One major constraint of this process, as well as of all the light-driven oxidation processes, is the formation of oxidation products deriving from the dissolved effluent organic matter (dE_fOM), which may induce toxic effects [5]. The dE_fOM present in biologically treated urban wastewater, consists of a heterogeneous mixture of refractory organic compounds with diverse structures and varying origin, including dissolved natural organic matter, soluble microbial products, endocrine disrupting compounds, pharmaceuticals and personal care products residues, disinfection by-products, metabolites/transformation products and others [6].

Granular activated carbon (GAC) has been widely tested in wastewater remediation for the removal of various CECs due to its ability to adsorb organic contaminants [7]. The efficiency and life-time of GAC depend however on several parameters, such as the quality of the effluent to be treated, the type of GAC used and the contact time. This makes the direct application of GAC after the biological treatment economically non-feasible, as the effluent and the dE_fOM contained, could exhaust GAC in a short period of time, imposing thus the application of extensive amounts of GAC and long contact times for the wastewater decontamination. Therefore, the application of GAC as a post-treatment to SPF, when the treated effluent contains reduced organic load and simpler organic molecules, could lead to the cost-efficient further remediation of the wastewater.

Within this context, the overall objectives of this study were two-fold: (a) to investigate the efficiency of SPF process in removing antibiotic resistance determinants and toxicity from urban wastewater (i.e. *(i)* degradation of selected antibiotics when present as a mixture in secondary-treated effluent, *(ii)* evaluation of phyto- and eco-toxicity of the treated effluent towards three plant species and *Daphnia magna*, respectively, *(iii)* evaluation of the disinfection potential of the process as to the inactivation of frequently encountered bacteria in wastewater, including those harbouring resistance to selected antibiotics [faecal coliforms, *Pseudomonas aeruginosa*, enterococci, total heterotrophs], as well as their regrowth potential after treatment, *(iv)* evaluation of the capacity of SPF to remove selected ARGs [e.g. 16S rRNA, bla_{TEM}, *tet*M, *sul*1 etc.]), and (b) to investigate the capacity of GAC, applied as post-treatment to SPF, to remove residual ecotoxicity and antibiotics not completely degraded during the early stages of SPF. The results of this study could contribute to the understanding of the fate of both the antibiotics and antibiotic resistance during advanced wastewater treatment and provide guidance for the practical application of the SPF-GAC process. According to the authors' knowledge, this is the first study investigating the combination of SPF and GAC processes, evaluating its efficiency in removing selected antibiotics, bacteria, ARB, ARGs, and toxicity from urban wastewater. This work suggests the SPF-GAC process could be a comprehensive solution, being not only able to cope with the toxicity problem of SPF, but also to provide in a short time, total removal of microcontaminants enabling the safe disposal of treated urban wastewater to the environment.

2. Experimental

2.1. Reagents

All antibiotics tested (ampicillin, clarithromycin, erythromycin, ofloxacin, sulfamethoxazole, tetracycline and trimethoprim) were of > 90% purity (Sigma-Aldrich) and were spiked in the effluent as a mixture (100 μg/L each) from individually prepared stock solutions (5000 mg/L). The stock solutions were kept in refrigerator and their stability was routinely checked through chromatographic analysis. During one-month period no statistical differences were observed. Iron (II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$, Sigma-Aldrich) and hydrogen peroxide (H_2O_2 30% w/w, Merck) reagents, were used for the SPF experiments.

For the adsorption experiments, GAC (Norit® ROZ3) purchased from Andreas Jacovides Trading Ltd (Cyprus), was used. The main physicochemical properties of the GAC can be seen in the Supplementary Information (**Table S1**).

2.2. Treated wastewater

Photocatalytic and adsorption experiments were performed using two different effluents, collected after the biological treatment of two Urban Wastewater Treatment Plants (UWTPs) in Cyprus. The first effluent was collected just after the sedimentation tank of a conventional activated sludge (CAS) system ($UWTP_1$: 151000 population equivalents [PE]; 21480 m^3/day; hereafter referred to as CAS effluent), and the second effluent was collected after the microfiltration membranes of a Membrane Biological Reactor (MBR) system ($UWTP_2$: 202000 PE; 9000 m^3/day; hereafter referred to as MBR effluent). The physicochemical characteristics and the concentrations of the target antibiotics detected in the CAS and MBR effluents are shown in **Table S2**. The exact protocol followed for the determination of the concentration of antibiotics is provided in **Text S1**.

2.3. Experimental procedures

2.3.1. SPF experiments

A compound parabolic collector pilot plant (total volume = 100 L, irradiated volume = 21.4 L) was used to perform the photocatalytic experiments [8]. A UV radiometer (CUV5, Kipp&Zonen) installed at the local latitude (35°), was used to record the solar irradiation during the process. The solar irradiation data were compared on different days and at different times of the day under different solar irradiation conditions using the "normalized illumination time", Eq. (1) [9]:

$$t_{30W,n} = t_{30w,n-1} + \Delta t_n \frac{UV}{30}\frac{V_I}{V_T} \quad \Delta t_n = t_n - t_{n-1} \tag{1}$$

where $t_{30W,n}$ and $t_{30W,n-1}$ are the adjusted experimental times according to UV energy (kJ/L) at experimental times t_n and t_{n-1}, respectively, Δt_n is the experimental time between two samples, V_I and V_T represent the irradiated and total volume, respectively, and UV is the average incident solar ultraviolet irradiation ($\lambda < 400$ nm) measured between two experimental times. The value of 30 refers to a constant solar UV power of 30 W/m^2, which corresponds to the typical solar UV power of a sunny day around noon. The value of 30 W/m^2 was used as the average value for the ultraviolet solar irradiation according to the UV data provided directly by the UV radiometer which is installed on the site. The collected UV data were confirmed with the data provided by the Cyprus Meteorological Service. It is important to mention that the normalized illumination time, calculated using the Eq. (1) is lower than the actual experimental time (t_{exp}). This is because the Eq. (1) include the term V_I/V_T and the irradiated volume is significantly lower than the total volume of the treated wastewater.

After the preparation of all the chemicals and reagents, the reactor, still covered to avoid any reaction before the beginning of the experiments, was filled with the effluent (60 L) and after a homogenisation of 10 min in the dark, the mixture of antibiotics was added. At that point, a sample was taken to measure the initial antibiotic concentration, and the appropriate amount of H_2SO_4 (3 M, Sigma-Aldrich) was added to adjust the pH to 2.8-2.9. Experiments were also performed at the inherent pH of the wastewater (pH_0 = 7.5–8.0) without the addition of H_2SO_4. The appropriate doses of ferrous iron (5 mg/L) and H_2O_2 (25–125 mg/L) were then added, the reactor was uncovered, and the SPF began.

Immediately after the collection of the samples, the Fenton reaction was terminated by adding methanol when chromatographic analysis

was to be performed. Methanol was reported to rapidly react with hydroxyl radicals and has been used extensively as $HO^{\cdot}$ scavenger to determine the presence and role of $HO^{\cdot}$ in photocatalysis [10]. For this reason, and also to be compatible with the analytical chromatographic method employed to determine the concentration of the antibiotics, which consisted of 10% methanol and 90% water, methanol was chosen to be used to stop the Fenton reaction. For the dissolved organic carbon (DOC) analysis, the reaction in the SPF-treated samples was terminated by adding anhydrous sodium sulfite (Na_2SO_3, Sigma-Aldrich). For the chromatographic analysis, formic acid (CH_2O_2, Fluka) of LC–MS grade was also used. For toxicity and microbiological analyses, the SPF-treated samples were neutralized with sodium hydroxide (NaOH 2 M, Fluka), while the residual H_2O_2 was removed with commercially available solution of bovine liver catalase (≥30,000 units/mg protein, Sigma-Aldrich). The determination of H_2O_2 during the SPF was performed using ammonium metavanadate, as described elsewhere [11] while its presence in the samples was also checked using Quantofix® strips (Sigma-Aldrich).

2.3.2. Adsorption on GAC

In order to determine the capacity of GAC in removing the target antibiotics from the effluents, batch adsorption experiments, applying different concentrations of adsorbent (500, 1000, 5000, 10,000 and 15,000 mg/L), were performed. The assessment was conducted using CAS and MBR effluents at their inherent pH (7 and 8, respectively). The experiments were performed in 300 mL glass beakers with continuous stirring and at a constant temperature of 25 °C. The initial concentration of antibiotics added, was 100 μg/L each. The samples were collected at various time intervals, filtered to remove the suspended GAC particles and subjected to further analysis. In all adsorption experiments performed, the removal of antibiotics followed the Freundlich model, as was confirmed by the linear expression of $\ln q_e$ vs. $\ln C_e$, Eq. (2):

$$\log q_e = \frac{1}{n} \cdot \log C_e + \log K_F \quad (2)$$

where q_e is the adsorption capacity (μg of adsorbate/mg of adsorbent) and C_e is the final equilibrium concentration of antibiotic in the liquid phase (μg/L). K_F constant characterizes the adsorption capacity, whereas 1/n is related to the intensity of the adsorption force between the activated carbon surface and the adsorbate.

2.3.3. Combined SPF-GAC experiments

In order to assess whether adsorption onto GAC is able to eliminate toxicity generated during SPF and also to remove antibiotics not yet degraded at the early stages of SPF, samples collected after 30, 60, 90, 120 and 180 min of SPF were subjected to adsorption on GAC (500–1000 mg/L). It was considered interesting to investigate the contribution of GAC to these samples, since at these times increased toxicity was observed and also significant antibiotic degradation (> 45% for all antibiotics) had been achieved. Immediately after collection, the pH of the samples was adjusted to 7 and the residual H_2O_2 was removed either with methanol or catalase, depending on the type of analysis to be performed. Samples were subsequently filtered through 0.45 μm Glass Fiber (GF) membrane filters, to remove the iron hydroxide formed at pH = 7, and then the appropriate amount of GAC was added. Here, it should be noted that the concentration of the target antibiotics prior to and after the neutralization-filtration of the SPF-treated samples, was measured and found the same, indicating no interference of the methodology to the removal of antibiotics. Adsorption experiments were carried out at neutral pH. Since the SPF-treated samples had to be neutralized immediately after their collection, for the catalase to act and stop the Fenton reaction, the subsequent adsorption experiments were carried out without modifying again the conditions. The experiments lasted for 180 min with gentle stirring. Samples were withdrawn at specified time intervals, filtered through 0.22 μm PES filters (Agilent), and further analysed for their content in antibiotics and ecotoxicity.

2.4. Analytical methods

The concentration of antibiotics in the samples collected throughout the processes, was monitored with a UPLC-MS/MS system (Waters) using a method specifically developed for this application (**Text S2, Table S3**).

DOC was measured using a TOC analyser (Aurora 1030). COD determination was performed using the Merck Spectroquant® kits.

The monitoring of total iron residues was performed according to the ISO method 6332, which makes use of 1, 10 phenanthroline (Fluka). The photometric measurements were performed using a double beam UV–Vis Jasco V-530 spectrophotometer.

2.5. Toxicity assessment

Toxicity measurements were carried out in samples collected during SPF and GAC processes, using: (a) Phytotestkit and (b) Daphtoxkit tests (MicroBioTests Inc.). Phytotoxicity of the treated samples was assessed towards three plant species i.e. *Sorghum saccharatum*, *Lepidium sativum* and *Sinapis alba*, and ecotoxicity tests were conducted according to the standard operating protocols for *D. magna* (ISO 6341:1996). The procedures are described extensively elsewhere [5].

2.6. Enumeration of total and antibiotic-resistant bacteria

Bacterial quantification was performed by the membrane filtration method. Different selective media were prepared for the enumeration of each type of microorganism examined; m-FC, Enterococcus Selective, Pseudomonas Agar Base, and PCA (Fluka) were prepared for the enumeration of faecal coliforms, *Enterococcus* spp., *P. aeruginosa,* and total heterotrophs, respectively. To evaluate the disinfection potential of SPF treatment, experiments were performed at the optimum experimental conditions, and samples collected through the treatment, were then plated on the appropriate, selective for each species agar, either in the presence or the absence of an antibiotic. For the quantification of ARB, the media were spiked with antibiotics (8 mg/L of erythromycin, 8 mg/L of ofloxacin, and 16 mg/L of trimethoprim) before solidification. These concentrations were chosen based on the minimum inhibitory concentration (MIC) of the antibiotics to different family of bacteria [12]. Serial dilutions were prepared in saline solution (NaCl, 0.85%) and filtered through membranes (mixed cellulose ester, 0.45 μm pore size, Millipore). The membranes were placed onto the culture media and incubated according to the optimum growth conditions for each species. The detection limit for faecal coliforms and *P. aeruginosa* was 5 and 3 CFU/mL respectively, while for *Enterococcus* spp. was 4 CFU/mL.

The regrowth of bacteria after treatment was examined by storing the treated samples in the dark for 24 h (25 °C) and their subsequent re-incubation on plates. The counted colonies during the regrowth experiments were considered as regrowth of damaged/inactivated bacteria that were previously unable to grow on the selective medium. Therefore, bacterial numbers during the regrowth test exceeding the bacterial numbers encountered during the treatment, were considered as repaired/reactivated bacteria that were previously damaged/inactive and were unable to grow on solid media (non-cultivable) due to the photocatalytic treatment.

2.7. Determination of total genomic DNA and ARGs

During the SPF process, samples were collected and stored at 4 °C in the dark. Molecular analysis was performed within 24 h after sampling. The DNA of the samples was extracted using the PowerWater® DNA isolation Kit (MoBio) following filtration of the samples with 0.22 μm polycarbonate filter membranes (Millipore). The amount of DNA in the extracts was measured using the Qubit fluorometer (PEQLab

BioTechnology).

Specific primers were used to quantify ARGs and opportunistic bacteria in total DNA (**Table S4),** during quantitative PCR analyses (qPCR). A real time qPCR assessment was carried out using the Bio-Rad CFX 96 Touch™ Real-Time PCR Detection System. The exact analytical procedure followed, is provided in **Text S3**.

3. Results and discussion

3.1. SPF experiments

3.1.1. Antibiotics degradation

In order to determine the optimum H_2O_2 dose, in which sufficient degradation of antibiotics could be accomplished during SPF experiments, several oxidant concentrations (ranging from 50 to 125 and 25–75 mg/L for CAS and MBR effluents, respectively) were tested. The range of H_2O_2 concentrations examined was based on the findings of previous studies [5,13]. The optimum concentration was found to be 100 and 50 mg/L of H_2O_2 for CAS and MBR effluents, respectively (*results not shown*). The need for higher concentration of oxidant in the CAS effluent was attributed to the higher concentration of dE_fOM, which may act as hydroxyl radicals' scavenger [6].

Fig. 1 depicts the degradation of antibiotics during the SPF treatment of CAS and MBR effluents, under the optimum experimental conditions in both acidic and inherent pH. In all the experiments performed, degradation of antibiotics exhibited a pseudo-first-order kinetics pattern, as was confirmed by the linear expression of $-\ln(C/C_0)$ (where C_0 and C refer to the concentrations of the antibiotics at times 0 and t (min), respectively), as a function of the treatment time. The apparent rate constant k_{app} was obtained from the slope of the linear plots and it is shown in the inset graphs of Fig. 1.

During treatment of the CAS effluents, four out of seven antibiotics (ampicillin, ofloxacin, trimethoprim and tetracycline) were completely degraded after $t_{30W,n}$ = 63 min, with ampicillin exhibiting the highest degradation rate among them (k_{app} = 0.083 min^{-1}). Erythromycin and clarithromycin on the other hand, needed more treatment time for complete degradation ($t_{30W,n}$ = 115 min), revealing their strong persistence towards SPF. Among the examined antibiotics, clarithromycin exhibited the lower degradation rate (k_{app} = 0.014 min^{-1}). Similarly, in the MBR effluents, ampicillin, tetracycline and trimethoprim were completely degraded after $t_{30W,n}$ = 40 min of treatment, while erythromycin, ofloxacin and sulfamethoxazole needed $t_{30W,n}$ = 79 min of treatment for complete degradation. Clarithromycin was not completely degraded even after $t_{30W,n}$ = 111 min of treatment. The behaviour of macrolide antibiotics may be related to their high molecular weight and their saturated structure, making thus their degradation difficult [14]. This behaviour has been previously observed by other investigators [15] who showed that clarithromycin has the lowest degradation rates during SPF, out of all the compounds investigated. The DOC of the reaction solution (corresponding to the inherent DOC of the wastewater that includes the low concentration of the spiked substrates and of their corresponding transformation products) decreased by only 10% in the case of CAS effluent and 22% in the case of MBR effluent. This may be ascribed to the higher organic content of the CAS effluent compared to the MBR effluents ($[DOC]_{CAS}$ = 10; $[DOC]_{MBR}$ = 6 mg/L). The relatively low yield of DOC removal compared to the substrates' depletion clearly points out the formation of recalcitrant organic intermediates deriving from the oxidation of the dE_fOM.

Experiments were also performed at the inherent pH of the wastewater (pH_0 = 7.5–8.0) to examine whether SPF is effective without acidification of the effluents, which would mean a reduction of the operating cost. As expected, the degradation of antibiotics was considerably hindered under inherent pH conditions, since there was no degradation of most of the antibiotics within 120 min of treatment (Fig. 1b–d). A significant decrease has also been observed in the k_{app} for all the antibiotics. During SPF performed at pH = 7, part of the

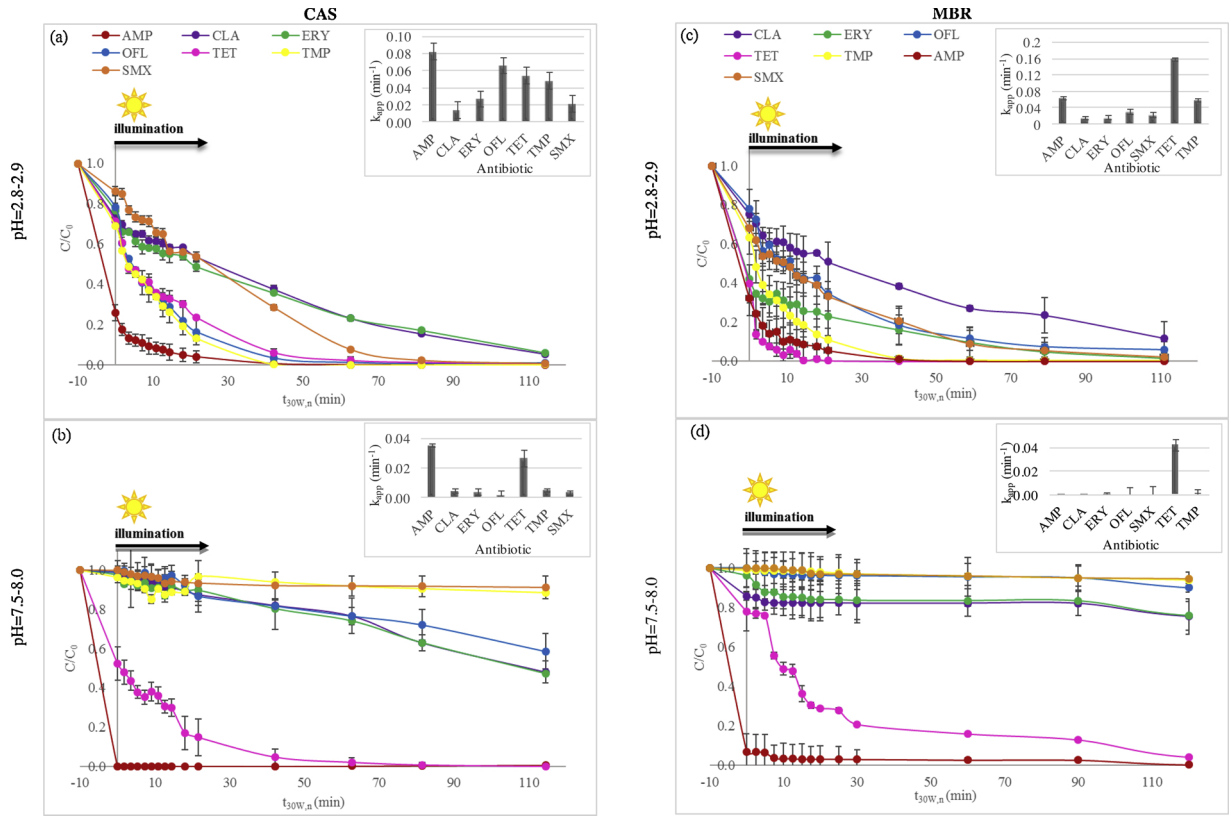

Fig. 1. Antibiotics degradation during SPF treatment of the wastewater effluents in acidic and inherent pH. *Experimental conditions:* $[A]_0 = 100\ \mu g/L$; $[Fe^{2+}]_0 = 5$ mg/L; $[H_2O_2]_{0,a,b} = 100$ mg/L; $[H_2O_2]_{0,c,d} = 50$ mg/L; $pH_{0,a,c} = 2.8–2.9$, $pH_{0,b,d} = 7.5–8.0$.

dissolved ferrous iron precipitates as iron hydroxide, while part of it is complexed with dE_fOM, resulting in a reduction of the overall performance of the process [5]. An interesting observation however, was that some antibiotics (ampicillin and tetracycline in both CAS and MBR effluents) were completely degraded under inherent pH conditions. Similar observations were also made in the study of Ioannou-Ttofa et al. [16], where complete degradation of ampicillin took place in the first 5 min of solar photo-Fenton at the inherent pH of the wastewater (7.5–8.0). The effect of pH on ampicillin degradation was associated with several non-determined complex reactions, related to the ionization state of the compound at inherent pH and to the existence of the molecule in its deprotonated form [17]. The degradation of tetracycline at this pH can be attributed to the photolysis of the compound, which is highly pH-dependent and strongly enhanced at high pH value [18]. During the dark period of the oxidation at the inherent pH conditions, the removal of ampicillin was observed to be 100% while that of tetracycline was approximately 50%. A similar observation was again made in the study of Ioannou-Ttofa et al. [16], where a ~95% of removal of ampicillin took place during the dark period of Fenton (at both the acidic and the inherent pH 7.5–8.0 of the wastewater). This is an indication that ampicillin is mostly affected by the Fenton reagent and not the pH. This is also supported by the fact that the concentration of ampicillin was assessed before and after the acidification of the wastewater which remained the same (*data not shown in the manuscript*). The latter stands also true for tetracycline.

DOC removal, during the SPF performed in acidic conditions, was similar or slightly increased compared to that observed under neutral pH conditions (8% and 10% in CAS and MBR effluents, respectively). Thus, it can be inferred that the pH conditions do not determine the overall process efficiency in terms of organic load removal.

3.1.2. Toxicity evaluation

Since several transformation products can be formed during SPF, resulting from both the oxidation of the antibiotics and the oxidation of the dE_fOM, the evaluation of the toxicity in the SPF-treated samples, was an important pillar of this study.

The SPF experiments were performed at the optimum experimental conditions, in both effluents, in the absence and in the presence of antibiotics (to distinguish whether the toxicity is generated from the oxidation of the dE_fOM or the target antibiotics). The results clearly indicated that the SPF-treated samples induced the same variation in the toxicity profile, implying that both the inhibition of the plants' growth and the immobilization of *D. magna* were neither affected by the presence of the parent compounds nor by their transformation products (*not shown*). Therefore, the toxic effects observed can be ascribed to the dE_fOM and its associated oxidation products.

3.1.2.1. Phytotoxicity assessment. SPF treatment displayed a varying toxicity profile for each type of plant in each type of effluent (**Figure S1**). While the germination of the seeds was the least affected, the growth of both the roots and shoots of the plants was significantly inhibited by the treatment. This was more pronounced in the case of *S. saccharatum*, which exerted the highest sensitivity compared to the other plants. These findings clearly demonstrate that the assessment of the potential biological potency of dE_fOM after SPF should not be overlooked, especially in the case where wastewater is intended for reuse.

Similar phytotoxicity results were obtained by Michael et al. [5] where SPF treatment of wastewater effluents induced toxicity against the three examined plant species but at the end of the treatment, a significant reduction of both root and shoot inhibition was observed (i.e. up to 60% reduction of root inhibition and up to 30% reduction of shoot inhibition in the initial wastewater sample).

3.1.2.2. Ecotoxicity assessment. The effluents, collected either after the CAS or the MBR system, resulted in an immobilization of 38% and 63% of *D. magna,* respectively, after 24 h of exposure time (**Figure S1**), indicating that the constituents of dE_fOM originally present in the effluents induce ecotoxic effects. After 48 h of exposure, the toxicity of the samples induced 53 and 100% of immobilization. From the beginning of the SPF treatment, the immobilization of daphniids in both matrices was either increased or maintained at similar to the initial high levels of untreated wastewater. In all samples examined, at 48 h of exposure, the toxic properties were found higher, and the immobilization reached a maximum value (77%–95%, at 30 and 60 min of treatment). This clearly indicates the toxic effects of the transformation products generated from the oxidation of the dE_fOM.

According to the authors' knowledge, there is limited available literature dealing with the toxicity effects of the dE_fOM oxidation products formed during SPF since only Michael et al. [5] so far reported the increase of toxicity during the SPF and ascribed it to the oxidation of the dE_fOM.

3.1.3. Enumeration of total and ARB

The total colony counts (including both, antibiotic-resistant and antibiotic-susceptible colony counts) for selected bacteria were enumerated in the treated wastewater. The inherent bacterial concentrations in the CAS effluents were: faecal coliforms $9.33 \times 10^2 \pm 4.50 \times 10^1$, *Enterococcus* spp. $5.57 \times 10^1 \pm 25.25 \times 10^0$, *P. aeruginosa* $1.87 \times 10^3 \pm 9.98 \times 10^2$, and total heterotrophs $8.27 \times 10^4 \pm 5.28 \times 10^3$ CFU/mL. As expected, after MBR treatment encompassing microfiltration membranes, the bacterial were very low: faecal coliforms $1.10 \times 10^1 \pm 1.0 \times 10^0$, *Enterococcus* $2.10 \times 10^1 \pm 3.0 \times 10^0$, *P. aeruginosa* $1.6 \times 10^2 \pm 2.8 \times 10^1$ and total heterotrophs $1.3 \times 10^3 \pm 1.7 \times 10^2$ CFU/mL. The antibiotic resistance patterns in the effluents were characterized from the ratio of ARB to the total colonies enumerated (**Figure S2**). Here the origin of antibiotic resistances were not determined (inherent or acquired). It was observed that the highest prevalence of all three types of bacteria examined was in the presence of trimethoprim, while the lowest prevalence of antibiotic resistance was in the presence of ofloxacin.

To evaluate the disinfection potential of SPF treatment, experiments were performed at the optimum experimental conditions for CAS effluents (i.e. $[Fe^{2+}]_0 = 5$ mg/L, $[H_2O_2]_0 = 100$ mg/L, $pH_0 = 2.8–2.9$) and samples collected through the treatment, were then plated on the appropriate, selective for each species agar, either in the presence or the absence of an antibiotic. It should be noted that, control samples prior to and after the acidification of the wastewater were collected, to investigate the effect of acidic pH conditions on the viability of bacteria. The results showed that an approximately 1-log reduction of the colonies took place after the acidification. This reduction is also obvious in Fig. 2, where the bacterial concentration prior to the acidification of the wastewater (at time -30) and after the acidification of the wastewater (at time -20) are presented. The treatment successfully inactivated all colonies, including colonies harbouring resistance to ofloxacin, trimethoprim and erythromycin. After 180 min of SPF there was a complete inactivation of all the selected cultivable bacteria, including the resistant ones in the CAS effluent (Fig. 2). As can be seen for *Enterococcus* spp., total heterotrophic bacteria and *P. aeruginosa*, a plateau is observed around the middle of the experimental period with no reduction of bacteria. It is noted that the concentration of iron remained stable throughout SPF, indicating no participation to the reactions. Therefore, this plateau can be correlated with the concentration of H_2O_2, which as observed in Fig. 2, its consumption remained stable during this time. According to Cadenas et al. [19] possible cause of this delay in disinfection could be that the natural bacterial population is resistant to the applied treatment, developing self-defence mechanisms against H_2O_2 and radiation induced damage. However, the disinfection rate increased at the final stages of the SPF treatment, a fact that can be confirmed by the complete removal of the total and ARB.

To determine the experimental time which is capable of permanently inactivating the bacteria, avoiding thus the re-activation and

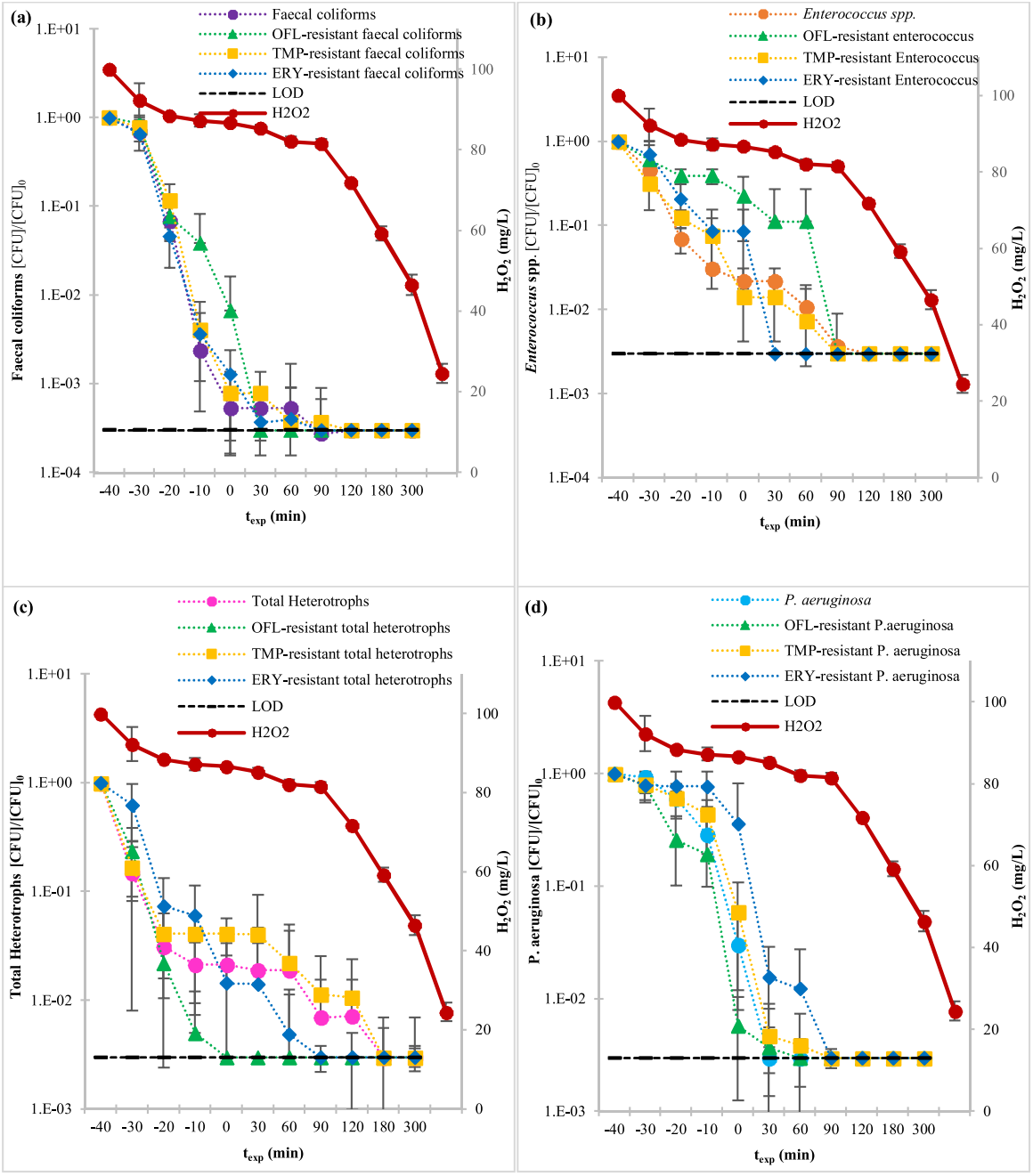

Fig. 2. Disinfection profile of total and antibiotic-resistant faecal coliforms (a), total heterotrophs (b), *Enterococcus* spp. (c) and *P. aeruginosa* (d), during the solar photo-Fenton treatment of the CAS effluents and consumption of H_2O_2 during the process. *Experimental conditions:* $[A]_0 = 100\,\mu g/L$; $[Fe^{2+}]_0 = 5\,mg/L$; $[H_2O_2]_0 = 100\,mg/L$; $pH_0 =$. 2.8–2.9.

regrowth of the bacteria during storage of the treated effluent, the samples which showed total inactivation of cultivable bacteria (90, 120, 180 and 300 min of SPF) were stored for 24 h in the dark (25 °C) and were then plated on the appropriate selective agar. No regrowth was observed in these tests, revealing that the mechanisms of action of SPF onto the cell metabolism have the potential to cause permanent damages.

It should be noted that because of the very low concentrations of bacteria in the MBR effluents, both the antibiotic resistance patterns and the inactivation profile of each species during SPF process, could not be determined using cultivation methods.

Similar results were obtained in a study by Karaolia et al. [13], where SPF process ($[Fe^{2+}] = 5\,mg/L$; $[H_2O_2] = 50\,mg/L$; pH 2.8) successfully inactivated antibiotic-tolerant *Klebsiella* spp., *P. aeruginosa*, and *Escherichia coli*. However, the authors observed a repair of *P. aeruginosa* 24 h after treatment, with 2 CFU/100 mL growing on the selective media, indicating their tolerance to the experimental conditions applied. This controversy may be attributed to the different culture media and enumeration methods used to determine regrowth of these bacteria. In a previous study of Karaolia et al. [15], SPF process applied at pilot-scale ($[Fe^{2+}] = 5\,mg/L$, $[H_2O_2] = 50\,mg/L$, pH = 4) was found able to reduce *Enterococcus* harbouring resistance to clarithromycin and sulfamethoxazole in wastewater. In this study, authors were referred to the radicals produced during the reaction which could destroy the cell wall of gram positive and negative bacteria. Ferro et al. [20], also showed that SPF ($[Fe^{2+}] = 0.09, 0.179, 0.358\,mM$, $[H_2O_2] = 0.588$,

1.470 and 2.205 mM, pH = 8.72) was able to eliminate *E. coli* resistant to ampicillin, tetracycline and ciprofloxacin in wastewater effluents.

3.1.4. Determination of total genomic DNA and ARGs

The total community DNA content of the treated wastewater was examined. The total genomic DNA concentration of the effluents at the beginning of the process before the SPF treatment, was 15.63 and 14.48 ng/μL in the CAS and MBR effluents, respectively **(Figure S3)**. In both effluents, during the loading of the reactor with the reagents on the preparation of the SPF experiment, a decreased total DNA content was observed (to a very low concentration of 5.15 and 0.16 ng/μL in CAS and MBR effluents, respectively). Continuing to 60 min of SPF treatment, the DNA content was reduced to the detection limit of the method (0.01 ng/μL), which accounts to a total DNA reduction of more than 99%.

The prevalence of 16S rRNA, *sul*1, *qnrS*, bla_{TEM}, bla_{OXA}, $bla_{CTX\text{-}M}$, and *tet*M ARGs was examined during the SPF treatment and the measured gene copies/100 mL DNA can be seen in Fig. 3. The abundance of the examined genes seemed to be altered during SPF. 16S rRNA, which is used as the standard for classification and identification of microbes, was the most prevalent genetic sequence detected in all samples examined (from 3.42×10^8 to 1.99×10^2 and from 1.72×10^8 to 4.91×10^1 copies/100 mL DNA in the CAS and MBR effluents, respectively). SPF successfully reduced 16S rRNA gene for about 6 logs, indicating a sensitivity of the bacteria population to the treatment. In both matrices, *qnrS* and *sul*1 were significantly reduced after the photocatalytic oxidation. These genes, although highly prevalent in human impacted environments, were originally at lower abundance than 16S rRNA genes and were reduced after 60 min of SPF in the case of CAS and before SPF begun in the case of MBR effluents, to levels below or close to the quantification limit (~10 gene copies/mL). During the SPF oxidation of the CAS effluents, bla_{TEM} was found to be the most prevalent among the tested ARGs, detected in all samples examined (1.96×10^4 - 1.6×10^1 copies/mL DNA), suggesting its lower susceptibility towards SPF conditions. Moreover, the abundance of these genes was shown to increase after 120 min of treatment (inset graph Fig. 3a). This may be attributed to the oxidative damage of bacteria during the first 60 min of SPF, causing them to release the specific gene, as well as a lower inter- and intra-species selective pressure [21]. As a result, a higher abundance and a lower removal was observed during the last 240 min of SPF. These findings are in agreement with Ferro et al. [22] who investigated the potential of a UV/H_2O_2 process to remove bla_{TEM} at a dose of 20 mg/L H_2O_2 in deionized water. The obtained results have shown no statistically significant reduction of the bla_{TEM} after 90 min of UV/H_2O_2 treatment.

In another study of Zhang et al. [23], the efficiency of dark Fenton and UV/H_2O_2 processes to reduce 16S rRNA, *intI1*, and selected ARGs (*sul*1, *tet*X, and *tet*G) present in wastewater effluents, was investigated under various operating parameters. The results obtained, showed that both processes could effectively reduce the selected ARGs, while Fenton was slightly more efficient. Under the optimum operating conditions (Fe^{2+}/H_2O_2 (mol):1/10; [H_2O_2] = 0.01 M; pH = 3) and reaction time of 120 min, 2.58–3.79 logs of target genes were removed.

3.2. Application of GAC

The results of toxicity towards *D. magna* during the SPF treatment (Section 3.1.2.2) clearly revealed that the oxidation of the dE_fOM present in wastewater may lead to toxic oxidation products. For this reason, SPF effluents were post-treated with GAC and its capacity to reduce the toxicity was investigated. In parallel, the contribution of adsoprtion onto GAC to the removal of antibiotics was also examined, at the optimum dose of GAC which eliminated ecotoxicity. In an attempt to reduce SPF application time, possibly minimizing this way the operational costs (by simplifying and shortening the process), the samples collected after 30, 60, 90, 120 and 180 min of SPF and subsequently post-treated with GAC (15 min of contact time with 500 mg/L of GAC) were also checked in terms of antibiotics' concentration. However, more info regarding the minimisation of cost, remains to be investigated on a pilot scale application.

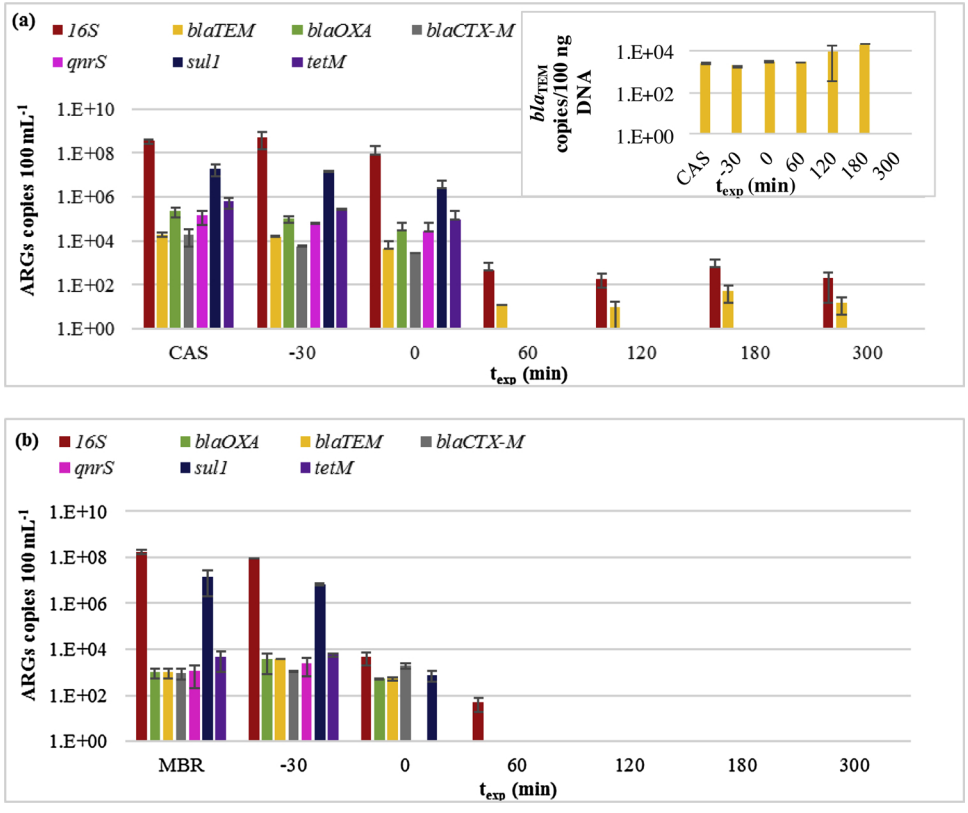

Fig. 3. Concentrations of ARGs (log copies per 100 mL of sample) in total DNA as a function of treatment time in SPF oxidation of CAS effluents (a) and MBR effluents (b). The inset graph depicts the concentration of blaTEM (log copies) in 100 ng of DNA. *Experimental conditions:* $[A]_0 = 100$ μg/L; $[Fe^{2+}]_0 = 5$ mg/L; $[H_2O_2]_{0,a} = 100$ mg/L; $[H_2O_2]_{0,b} = 50$ mg/L; $pH_0 = 2.8–2.9$.

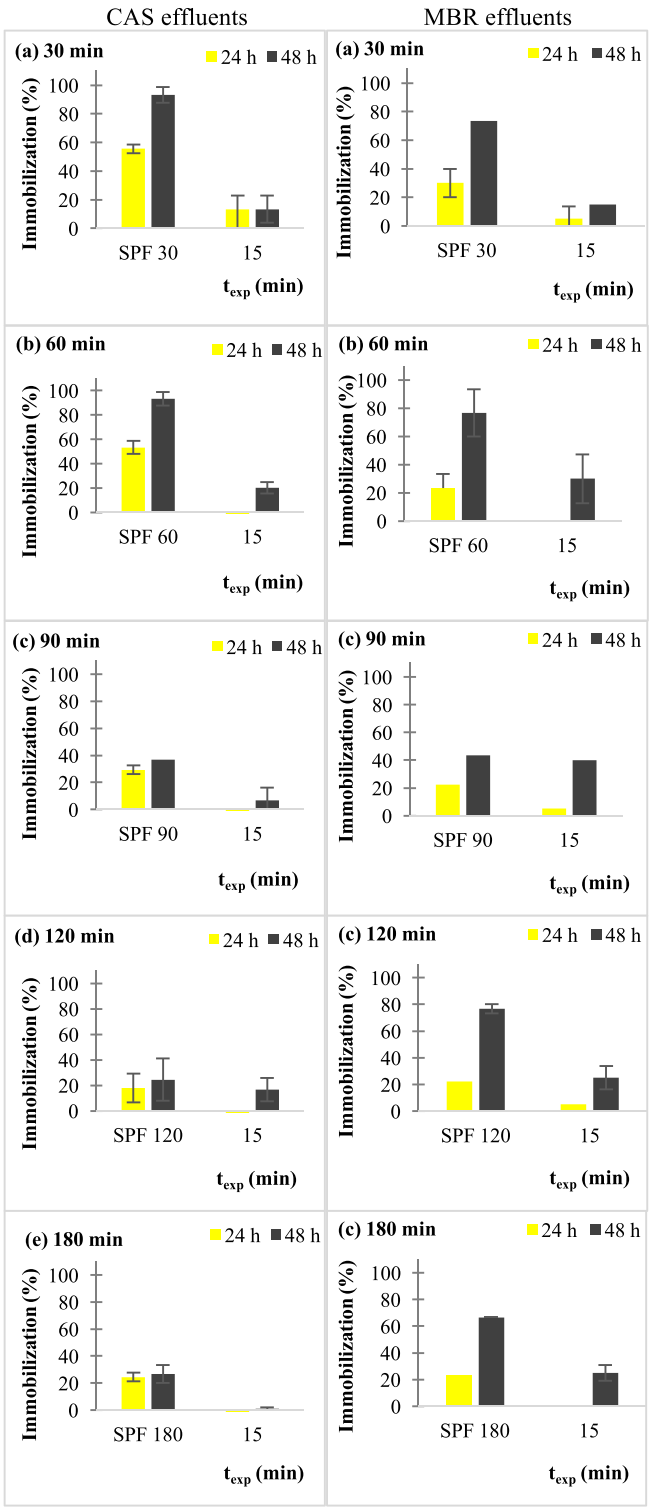

Fig. 4. Elimination of ecotoxicity towards *D. magna* during post-treatment of SPF treated flow with GAC. Samples were collected at 30 min (a), 60 min (b), 90 min (c), 120 min (d) and 180 min (e) of SPF treatment. Experimental conditions: [GAC] = 500 mg/L; pH_0 = 7; Temperature: 25 ± 2 °C, contact time = 15 min.

3.2.1. Toxicity assessment

As the toxic effects observed during the SPF treatment, were more intense towards *D. magna*, compared to those shown for the plants, it was decided to further study only the ecotoxicity effects. Therefore, the toxicity of the samples towards *D. magna* was measured before and after the application of two amounts of GAC (500 and 1000 mg/L) with 15, 30 and 60 min contact times (*data not shown*). The reduction of toxicity was accomplished, after 15 min of contact time using 500 mg/L of GAC (Fig. 4). The toxicity of all post-treated with GAC samples towards *D. magna*, was lower compared to that observed during SPF treatment, suggesting that the toxic oxidation products were adsorbed onto GAC.

According to the authors knowledge, this is the first study investigating the combination of SPF process with adsorption onto GAC, for the reduction of toxicity. Some studies were conducted on the application of ozonation combined with activated carbon adsorption. For example, in the study of Reungoat et al. [24], biological activated carbon filtration was used as post-treatment of ozonated samples and was found capable of further removing some of the oxidation products remaining after the process (by up to 99%) and also reducing the non-specific toxicity (Microtox assay) by up to 54%. Similarly, in the study of Bourgin et al. [25] GAC adsorption proved to be effective in removing transformation products remaining after ozonation, due to its high adsorption capacity.

3.2.2. Adsoprtion of antibiotics onto GAC

3.2.2.1. Adsorption capacity of GAC. The adsorption capacity of GAC was determined through the Freundlich isotherm (**Figure S4**). The data used to calculate the Freundlich coefficients resulted from the batch adsoprtion experiments performed employing different amounts of GAC (500–15000 mg/L) in CAS and MBR effluents which were spiked with the mixture of antibiotics (100 μg/L each). In general, the adsorption of all selected antibiotics was shown to follow the Freundlich model (**Figure S4**). Between CAS and MBR effluents, no significant differences on the Freundlich coefficients, were observed.

Regarding the efficiency of GAC to adsorb the selected antibiotics when present as a mixture in secondary-treated effluents, small variation, in terms of the removal extent of each antibiotic, was recorded (**Table S5**). The removal efficiencies of the antibiotics seemed to increase with increasing adsorbent quantity, whereas the highest removal of antibiotics was exhibited with the application of 15,000 mg/L of GAC (87%–98% removal). The concentrations of 500 and 1000 mg/L of GAC were insufficient to remove the antibiotics more than 40%. The physicochemical properties and competition effects of the antibiotics can affect the removal efficiency among the different compounds. For example, the lower molecular weight and smaller molecular volume of trimethoprim seemed to facilitate the adsorption of the compound onto the carbon, leading to higher removal. Compared to trimethoprim, the lower removal of ampicillin, clarithromycin and erythromycin was possibly due to their relatively larger molecular weight and complex structure. Therefore, it can be concluded that the differences of the physicochemical characteristics of the antibiotics contribute significantly to their different removal efficiencies in the GAC adsorption process. Accordingly, in the study of Snyder et al. [26] the breakthrough curves clearly demonstrated that the compounds with greater hydrophilicity breach activated carbon faster than hydrophobic compounds.

As can be concluded from the above, the application of GAC immediately after the biological treatment of wastewater to remove the tested antibiotics, requires long contact times (90 min) and high adsorbent concentrations (10–15 g/L). For these reasons, GAC is commonly used at many wastewater treatment plants as a replacement for anthracite media in conventional filters, thus providing both adsorption and filtration and alternatively, GAC can be applied in post-conventional filtration as an adsorbent bed [27,28]. During SPF treatment, degradation of antibiotics is taking place and concentrations of antibiotics are reduced (from 100 to 4–54 μg/L, in 30 min of SPF). Therefore, lower amounts of adsorbent can be applied during adsorption as post-treatment and yield to significant removals of antibiotic residues.

In an attempt to minimise the operating costs of the SPF, an effort was made to reduce the time of the application of the photocatalytic process. Thus, samples were collected during SPF, i.e after 30, 60, 90,

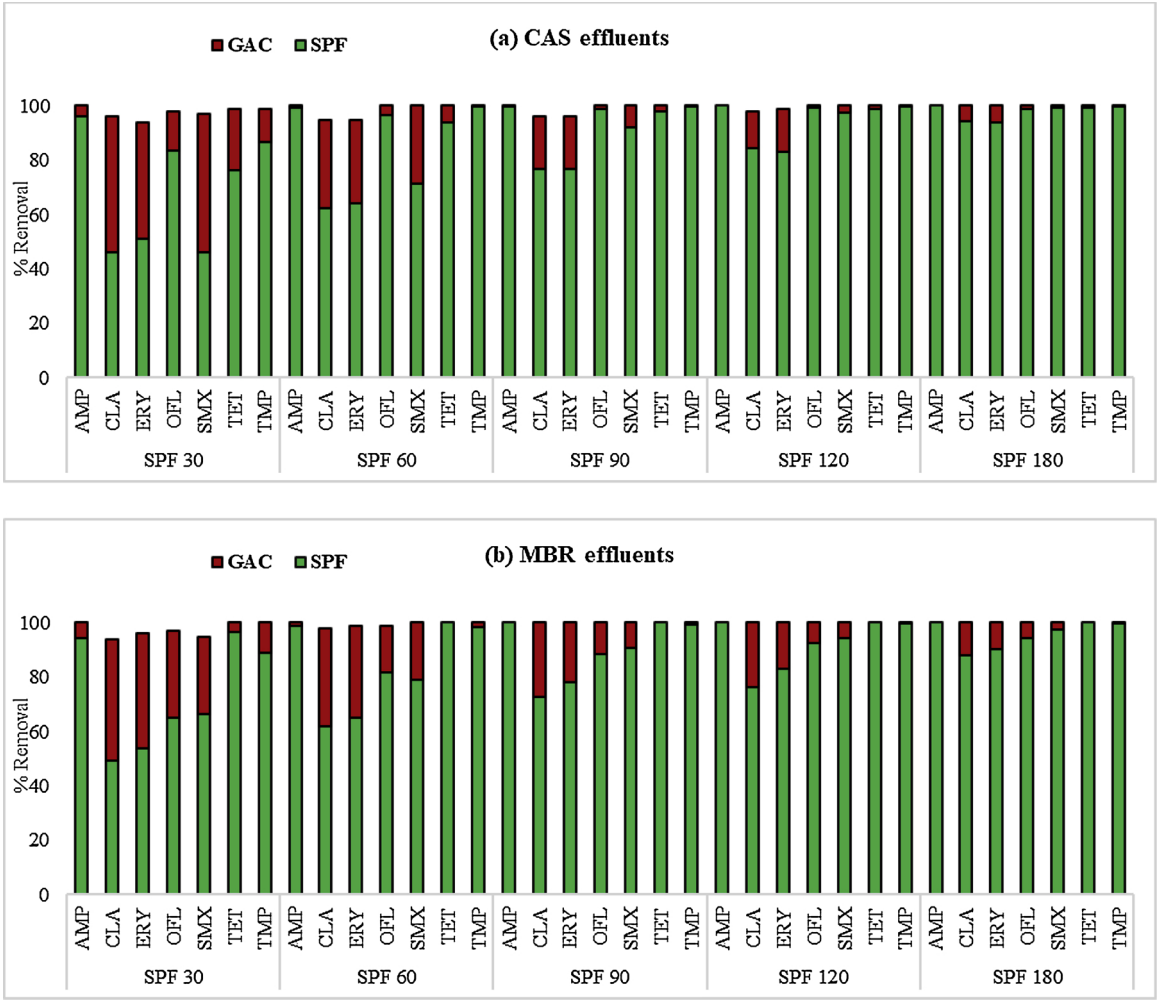

Fig. 5. Removal of antibiotics by SPF and SPF-GAC processes at different experimental times. *Experimental conditions:* $[A]_0 = 100$ μg/L; $[Fe^{2+}]_0 = 5$ mg/L; $[H_2O_2]_{0,a} = 100$ mg/L; $[H_2O_2]_{0,b} = 50$ mg/L; pH = 2.8–2.9, [GAC] = 500 mg/L; $T = 25 \pm 1.0$ °C, pH = 7.5–8.0, contact time = 15 min.

120 and 180 min and, were post-treated with GAC. Then, the effectiveness of the combined technology with regard to the removal of antibiotics, was assessed.

3.2.2.2. SPF-GAC antibiotics removal. The samples collected through SPF treatment were post-treated with 500 mg/L of GAC and the concentration of antibiotics was monitored for 180 min (*data not shown*). It was found that after 15 min of contact with GAC, the remaining antibiotics in SPF effluents were eliminated (Fig. 5). Although the addition of 500 mg/L of GAC in secondary-treated effluents inadequately removed antibiotics (**Table S5**), in the SPF-treated samples this concentration of GAC eliminated them, indicating that smaller concentrations of antibiotics can be better adsorbed onto GAC. Taking as an example sulfamethoxazole (with initial concentration of 100 μg/L in the secondary-treated effluents and a 67% of degradation by 30 min of SPF), an additional 28% removal is observed with 15 min of contact with GAC, which means 95% of total removal. In the case of clarithromycin and erythromycin, which had been degraded only by 46–51% respectively by 30 min of SPF, the addition of GAC seemed to have the largest contribution, since additional 50-43% removal was achieved, thus increasing the overall performance of SPF-GAC. Also, compounds that had been degraded more than 75% by 30 min of SPF (ampicillin, ofloxacin, tetracycline and trimethoprim), were shown to be completely removed after the post-treatment with GAC. As can be seen in Fig. 5, the removal efficiency of SPF-GAC process was > 95% for all the studied antibiotics. SPF here was shown to exhibit the dominant contribution to the removal of antibiotics, by reducing their concentrations to the half, whereas GAC acted as a beneficial supplement to completely eliminate them. Even though DOC of CAS effluent was higher than that of MBR effluent after 30 min of SPF, no significant difference in the extent of adsorption of antibiotics onto GAC was observed in the two effluents.

Overall, it is clear that SPF and GAC processes showed additive effects for the removal of the target antibiotics, and that GAC process is a necessary and beneficial additional step for the entire process.

4. Conclusions

The present study focused primarily on the minimisation of antibiotics in urban wastewater, while the fate of antibiotic resistance determinants during the SPF-GAC treatment, was also examined. The combination of SPF with adsorption was proved to be effective not only for the removal of antibiotic related microcontaminants but also for the elimination of toxicity in urban wastewater. Briefly, the main conclusions are:

SPF was found to be efficient in degrading antibiotics but the process resulted in increased toxicity of the treated samples. Also, SPF was successful in inactivating faecal coliforms, *Enterococcus* spp., *P. aeruginosa,* and total heterotrophs, including colonies carrying resistance to trimethoprim, ofloxacin, and erythromycin, individually, whereas it has provided complete and permanent disinfection of urban wastewater,

even after 24 h of post-treatment storage of the treated samples. ARGs exhibited different behaviour during SPF, as specific genes were completely degraded, while others, such as *bla*$_{TEM}$, were persistent throughout the treatment.

The application of GAC as a post-treatment to the SPF-treated flow was found to be capable of eliminating toxicity and antibiotics in the effluents. However, further research is needed to investigate, in addition to the effect of SPF, the contribution of adsorption process to the disinfection of urban wastewater and the fate of ARGs during the combined process. Considering that the SPF-treated flow is acidic, further investigations are required, possibly at a pilot-scale level, to thoroughly understand the effect of pH on the behaviour of antibiotic resistance determinants during the application of GAC, and to assess the extent this is related with the cost of the combined process.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.apcatb.2018.12.030.

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